INFLUENCE OF GROWTH HABIT, NODULATION, AND NITROGEN FERTILIZATION ON THE INCIDENCE OF SOYBEAN CHARCOAL ROT AND IN VITRO RESPONSE OF SOYBEAN CULTIVARS TO THE TOXIN OF *M. PHASEOLINA*, CAUSAL FUNGUS OF THE DISEASE

by

HONGRUN YU

B. S., Shenyang Agricultural College, China, 1981

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INTRODUCTION

Charcoal rot is an important fungal disease. The causal agent [Macrophomina phaseolina (Tassi) Goid] can infect more than 500 plant species (Sinclair, 1984). This host range encompasses 75 plant families from cereal, legumes, oilseeds, fiber crops, vegetables to trees, and fruit trees (Pearson, 1987). Among these are many economically important agricultural crops, such as maize, sorghum, millet, soybean, peanut, cowpea, chickpea, mungbean, sunflower, cotton, tobacco, potato, sweet potato, eggplant, pine tree, apple, pear, orange and banana.

It is estimated that crop losses due to charcoal rot are 10-15% worldwide each crop year (Dhingra and Sinclair, 1978). Fifty percent and up to 77% estimated yield losses in soybeans due to charcoal rot have been reported in Yugoslavia and India, respectively (Agarwal and Sarbhoy, 1976; and Meyer et al., 1974). In Kansas, charcoal rot is a major disease problem of soybeans. Charcoal rot occurs throughout the dryland soybean production areas of the state.

Soybean charcoal rot is described as a disease of roots and lower part of the stem (Athow, 1973). The disease may appear as a seedling blight or as a root rot (Meyer et al., 1973). Infected seedlings may show a reddish-brown discoloration at the emerging portion of the

hypocotyl. Root infection can result in discoloration at the soil line and above. The lesions turn dark brown to black, with the death of the seedlings under hot, dry conditions (Sinclair, 1984).

In older plants, the disease becomes apparent after midseason when plants are flowering. Light gray discoloration of the epidermal and subepidermal tissues in taproot and lower part of the stem can be observed. When the root is split, numerous black bodies exists in the xylem vessels and pith tissue. These are microsclerotia, asexual propogules of the fungus (Sinclair, 1983). Internal and external reddish brown lesions and sclerotia were also observed on older plants in the field throughout the growing season (Pearson, 1984). Leaves turn yellow, but remain attached (Sinclair, 1983). Symptoms also include premature senescence (Bowen, 1987).

The twin-stem abnormality caused by M. phaseolina has been reported on soybean seedlings (Bristow and Wyllie, 1986). Rosseting of soybean seedlings inoculated with the fungus also occurs (Dhingra and Sinclair, 1973).

In this disease system, toxin production is also involved. In the 1920's, Small observed disease symptoms well before the hyphae and suspected that toxic substances were produced (Dhingra and Sinclair, 1978). Since then, the toxin has bean studied by several authors. Chan and Sackston (1969) induced necrotic spots on sunflower plants

and the detached leaves using cell free culture filtrate. The pattern was similar to that produced on the leaves of inoculated soybean seedlings. The partially purified toxin from culture produced symptoms on the cut soybean seedlings (Dhingra and Sinclair, 1974). These included the terminal softening, vein browning, and necrosis of the stem and leaf tissues, followed by dehydration and defoliation. Symptoms were also observed on the cut seedlings placed in the toxin isolated from inoculated soybean seedlings. Symptoms produced from both sources were typical with those on the inoculated seedlings just before their death. Pearson (1982) subjected the cut soybean seedlings from 14 genotypes to the partially purified toxin. All seedlings showed desiccation after 15 hours.

Chan and Sackston (1969) used eosin dye, which was translocatable in plants, to induce coloration on soybean seedings. The coloration pattern was the same as that of necrotic spotting caused by the toxin. They concluded that the toxin was translocated in the vascular system. Systemic movement of the toxin was evidenced by the work of Dhingra and Sinclair (1974). Chan and Sackston (1973) tested 21 plant species against the toxin from four isolates of M. phaseolina and found the toxin was not host specific.

The toxin is soluble in water, aqueous pyridine,

methanol, and insoluble in organic solvents and thermostable (Chan and Sackston, 1969; Dhingra and Sinclair, 1974). It was suggested that it was neither protein nor enzyme. It might be a polar compound. The toxins isolated from both culture and plant tissues were chromatographically similar with absorption maxima at 225 nm (Dhingra and Sinclair, 1974). High performance liquid chromatography (HPLC) of trifluroacetic acid hydrolyzed toxin yielded several sugars, alpha-deoxyribose, rhamnose, mannose, fructose, fucose, galactose, xylose, glucose and two unidentified compounds (Pearson, 1982). The presence of protein was also suspected by Pearson. This mixture had a molecular weight of 2×10^6 (Pearson, 1982).

The dialyzed culture filtrate of M. phaseolina had antibiotic activity and was shown to inhibit several bacteria and fungi (Pearson, 1982). This was speculated as a tool of the fungus to maintain a food base through decreasing competition among microorganisms.

The disease symptoms are due to a series of factors (Dhingra and Sinclair, 1978). The major symptom is dehydration of leaves. Dhingra and Sinclair (1978) speculated that this mainly resulted from plugging by intraxylem sclerotia for older plants and from toxin for seedlings.

In the field, this disease is stress-related. The development of the disease is encouraged by many stress

factors (Sinclair, 1983). The disease usually becomes apparent when infected plants are under hot, dry and other stress conditions or in progressive senescence (Meyer et al., 1974; Sinclair, 1983). Dense plant populations also aggravate the disease (Bowen, 1987).

It has been found that early maturing cultivars tend to have higher infection and late maturity cultivars lower infection (Pearson, 1984; Bowen, 1987). Such a phenomenon was explained due to the late maturity cultivars flowering late to escape high temperature and scarce precipitation in mid-summer (Pearson, 1984). Soybean growth habit also influences the incidence of soybean charcoal rot (Bowen, 1987). Greater water demand during pod filling stage in indeterminate culyivars was suggested to be responsible for higher colonization by M. phaseolina.

Resistance breeding has been employed to deal with disease problems in many crops. Although differences in colonization by the fungus do exist (Agarwal and Sarbhoy, 1976; Schapaugh and Schwenk, 1980 unpublished), resistance has not been found (Sinclair, 1983). Nearly all cultivars studied were infected (Pearson, 1987; Bowen, 1987). Apparently, resistance breeding strategy can not be used to attack the disease until resistance is found by appropriate screening techniques. Unfortunately, no suitable techniques have been developed (Pearson, 1982).

Some control strategies may be useful to cope with the disease. M. phaseolina is an opportunistic microorganism. Plants under nutrient deficient conditions are more susceptible to the disease. Adequate and balanced soil fertility can reduce charcoal rot losses (Sinclair, 1983). Waterlogging decreases the number of sclerotia in soil (Dhingra and Sinclair, 1978). Flooding a field 3-4 weeks before planting for disease management was suggested by Sinclair (1983). The role of crop rotation is justified by the host selectivity and ecological specificity of M. phaseolina. Bristow and Wyllie (1975) found that plots rotated between soybean and corn had only half the inoculum density of M. phaseolina of continuous soybean plots. Root colonization by the fungus was also 33% more in the continuous soybean plots. Similar results were obtained by Pearson (1987). Continuous planting of corn or rotation between soybean and corn reduced the fungus population in the soil and the corn tissues. Rotation with corn may be a practical control method for the disease. Soybean charcoal rot is also a seed borne disease (Sinclair, 1984). Using good seed can be helpful in reducing the disease (Sinclair, 1983). Proper weed and insect control which reduces their competition with soybeans may also be useful (Sinclair, 1983). Several systemic fungicides have been tested or listed that can used as seed or soil treatment or for leaf spraying (Ilyas et al., 1976; Sinclair, 1983).

PART I INFLUENCE OF GROWTH HABIT, NODULATION AND NITROGEN FERTILIZATION ON THE INCIDENCE OF SOYBEAN CHARCOAL ROT

ABSTRACT

soybean cultivars of different growth habits were reported to have differential response to charcoal rot infections. Previous laboratory work showed that isolates of M. phaseolina, causal fungus of the disease, from soybean and corn tissues responded differently to chlorate, an analog of nitrate. Because soybean isolates were chlorate-sensitive, they might be also sensitive to the intermediates of nitrate metabolism. Both field and greenhouse studies were conducted to investigate the influence of growth habit, nodulation and nitrogen fertilization on the non-nodulated isolines on the root colonization in soybeans by the chlorate sensitive fungus of M. phaseolina with near-isogenic lines of growth habit and nodulation of Clark and Harosoy. Charcoal rot infection was determined by LOG10CFU and color rating obtained from the ground roots. There was no significant difference between the determinate isoline and the indeterminate isoline in LOG10CFU and color rating. But, the determinate plants consistently had less LOG10CFU and color rating in both field and greenhouse studies. Nitrogen fertilization reduced charcoal rot infection when charcoal rot infection

was measured by color rating and increased soybean yield on the non-nodulated isolines. Nodulation increased seed yield compared with the unfertilized non-nodulated isolines. The influence of nodulation on the root colonization by M. phaseolina in soybeans was erratic. The nodulated isolines had similar or slightly higher levels of charcoal rot infection in the field study and lower infection in the greenhouse study than the unfertilized non-nodulated isolines.

Charcoal rot is an important fungal disease for soybeans in the mid-west United States. Recent studies have shown that many factors influence soybean charcoal rot infection (Pearson, 1984, 1987; Bowen, 1987). Soybean growth habit was reported to affect charcoal rot development (Pearson, 1984; Bowen, 1987). Indeterminate plants tended to have higher root colonization by M. phaseolina, casual agent of the disease, than determinate plants. After examining water usage of determinate and indeterminate soybean plants, Bowen (1987) suggested greater water demand during pod fill may be responsible for the increased infection in indeterminate soybean plants. However, the difference between determinate and indeterminate soybean plants in terms of charcoal rot infection might be confounded by the genotypic difference of the cultivars the two authors used. The first objective of this study was to study whether the same tendency occurred under field and greenhouse conditions using soybean near-isogenic lines for growth habits.

A series of experiments conducted by Pearson (1986) indicated that isolates of M. phaseolina, from corn, another host, were chlorate-resistant and showed the dense regular growth pattern on medium containing potassium chlorate. Isolates from soybean tissues and soybean field soil were sensitive to chlorate and displayed either feathery or restricted growth patterns in the presence of

potassium chlorate. As nitrate is reduced to nitrite by nitrate reductase, chlorate is also reduced by this enzyme to chlorite. Generally speaking, only chlorate-sensitive isolates can carry out the reduction of nitrate to nitrite, while the chlorate-resistant isolates can not. It is suspected that the excessive amount of nitrogen available to the chlorate-sensitive fungus in soybeans might lead to the accumulation of such intermediates as nitrite and hydroxylamine, which are toxic to the fungus. Therefore, nitrogen might reduce charcoal rot infection in soybeans. On the other hand, when tested on the nitrate or nitrite medium, the chlorate-sensitive soybean isolates surprisingly grew well compared with chlorate-resistant corn isolates (Pearson, 1987). Such conflicting results of nitrogen effect on the fungus led to the hypothesis that in addition to its influence on M. phaseolina, nitrogen may also modify the defense mechanism and the suitability as substrate of soybean plants to the fungus. The second objective of this study was to investigate the effect of nodulation, with near-isogenic isolines, and nitrogen fertilization on the non-nodulated isolines on the root colonization by this fungus in soybeans under greenhouse and field conditions.

MATERIALS AND METHODS

Field Experiments

The experiment was conducted at two locations in Kansas, Hesston and Parsons, in 1987. The Hesston location was planted on May 10. At Parsons, there were two plantings, one on May 22 and the other on June 10, designated as Parsons1 and Parsons2, respectively. A randomized, complete block design with 3 replications was used in the field known to be naturally infested with *M. phaseolina*. Each plot consisted of 4 rows spaced 0.76 m apart and 6.10 m long. After end trimming at maturity, the two center rows were harvested for yield determination and the two outside rows were used for root sampling.

The ten entries were Harosoy (Group II), the fertilized and unfertilized treatments of L65-1274 (non-nodulated isoline of Harosoy), Sprite (Group III), Williams 82 (Group III), Clark-L1 (Group IV), the fertilized and unfertilized treatments of L63-1889 (non-nodulated isoline of Clark-L1), L63-3016 (determinate isoline of Clark-L1) and Douglas (Group IV). Harosoy isolines were not present at Parsons2. For the fertilized treatment, 250 pounds of granular ammonium nitrate per acre were hand-applied to the surface of fertilization plots following seed sowing.

Four plants, two randomly selected from each of two outside rows, were taken as a sample for each plot at R7

stage. Roots were excised at the cotyledonary node. Root samples were maintained in the cold room (4 ^{o}C) until they were washed with tap water to remove dirt and nodules. They were then surface-sterilized in a 0.8% sodium hypochlorite (made from Clorox, a commercial bleach) and 5% ethanol solution for one minute and rinsed in distilled water. The blotted dried root sample was then put in a paper bag and dried in a forced-air oven at room temperature overnight for 15 hours. Samples were ground in a Wiley mill and passed through a one millimeter screen. The ground root sample was stored in a test tube.

Test tubes containing root samples from all environments were mixed together and put on a white paper on a desk. The darkest and the lightest ones were selected out. The remaining test tubes were arranged from the lightest to the darkest. Color scores were given to each root sample, the lightest 0 and the darkest 10 as described by Bowen (1987).

From 20 to 100 mg of each ground root sample was mixed in 100 ml of Cloroneb-mercury-rose bengal agar, a selective medium for M. phaseolina (Meyer, 1973) and poured into five 15x100 cm petri dishes. The plates were incubated in the dark at 30^{o}C for about one week and number of colonies was counted for each root sample. Colony forming units (CFU) were calculated on a per gram root sample basis.

CFU was transformed into LOG10CFU statistically with

the formula $LOG10CFU=\log_{10}(CFU+1)$ (Dowen, 1987). Statistical analysis was done with GLM and ANOVA facilities of SAS software. Three analyses were conducted. The general analysis used the ten entries. Individual analyses were conducted on the growth habit isolines of Clark and the nodulation isolines of Harosoy and Clark. The nodulation isoline analysis included three treatments, the nodulated, the unfertilized and fertilized non-nodulated isolines. Significance of the environmental (ENV) effect was tested with the mean squares of replication within environment [REP(ENV)] as error term in analyses.

Greenhouse Experiment

The field study was also repeated in a greenhouse. Pots with soil brought from a field in Parsons known to be infested with M. phaseolina were used. Soil test indicated that the soil nitrate nitrogen content was 16 ppm, phosphorus was 42 pounds per acre and potassium was 250 pounds per acre. The pH was 6.5.

A randomized, complete block design with 4 replications was used. Four pots containing single plants for each entry were assigned into four replications occupying different regions on a bench. Each entry was randomly placed within each replication.

Seeds of the experiment were surface sterilized in a 0.8% sodium hypochlorite solution (made from Clorox, a

commercial bleach) for one minute and rinsed in tap water. Four seeds were planted in each 6 L pot (21 cm in diameter) on December 22, 1987. Seedlings were thinned to one plant per pot. For the fertilized treatment of the non-nodulated isolines, fertilizer was applied 20 days after planting. To achieve 30 ppm of nitrogen content on the fertilized treatment, 0.29 grams of ammonium nitrate with nitrogen content of 34% was dissolved in 200 ml water, and the solution was poured on the soil surface of each fertilized treatment pot.

Natural light was supplemented with sodium vapor lamps to provide a 14 hour photoperiod. The temperature was set for a maximum of 31 ^{o}C during the day and a minimum of 18 ^{o}C at night.

Drought stress was applied basically with the late treatment described by Bowen (1987). The plants were watered normally until 45 days after planting when the stress was initiated. Three hundred ml of water were given to each pot on every other and on every third day, alternatively. Eighty days after planting, 145 ml of water was applied to each pot on a daily basis. On rainy days, plants were watered on every other day to maintain the stress condition. Those plants that could not mature naturally were all harvested on May 6, 1988. Watering was withdrawn a week before their harvest.

During the growth period, the date of each reproductive

stage and the plant height at each reproductive stage were recorded for each plant. Eighty four days after planting and at R1-R3 stages, chlorosis scores (from 0 for normal green leaf color to 3 for severe yellow leaf color) were taken on each plant. Stem diameter was measured at the cotyledonary node 110 days after planting when most plants were at R6 stage. After plants were harvested, roots were excised at the node next to the cotyledonary node and maintained at 4 ^{o}C for processing. The above cotyledonary internode, shoots and seeds were oven-dried at 30^{o}C to a constant weight for obtaining dry shoot weight and dry seed weight. The cotyledonary internode was also weighed before drying and used for stem water content determination. The cotyledonary internode and the root of each plant were put together and then ground to form a ground root sample. The methods of root grinding, color rating of ground root samples and analyses were basically the same as for the field study. Roots were ground with 40 mesh screen for the greenhouse study. Instead of 0-10 color rating scale for the field study, 0-5 scores were used.

RESULTS AND DISCUSSION

Field Experiments

The analyses of variance and the means of charcoal rot data and major agronomic traits are given in Tables 1 and 2. The environmental effect was significant for LOG10CFU, plant height, and maturity. Harosoy isolines were not present at Parsons2. This may have resulted in part in the environmental differences for some traits. Analysis with cultivars that were present at all three environments indicated that late planting Parsons2 had a significantly higher LOG10CFU than Hesston and Parsons1, Parsons had a significantly higher color rating than Hesston (Table 3). Parsons2 also had a significantly higher yield and earlier maturity than Parsons1. Parsons1 had a significantly higher average plant height than Hesston and Parsons2.

Entry effects were significant for all traits studied (Table 1). Environment by entry interactions were significant for LOG10CFU, color rating and plant height.

Maturity was negatively correlated with LOG10CFU and color rating (Table 4). As maturity increased, LOG10CFU and color rating decreased (Table 2). This was especially apparent for the first planting data. Previous studies also suggested that early maturity cultivars tended to have more charcoal rot infection than the late maturity cultivars (Pearson, 1984; Bowen, 1987).

Yield was negatively correlated with color rating (Table 4). There was also a significant correlation between maturity and yield. One should be aware that the negative correlation between yield and color rating might be due to the significant correlations of maturity with both color rating and yield.

LOG10CFU and color rating were significantly correlated with each other. This supports the result that color rating agreed with LOG10CFU (Bowen, 1987). Since LOG10CFU was not significantly correlated yield, color rating seemed to a better measure of charcoal rot infection.

The Clark determinate isoline had less LOG10CFU at Hesston and for the combined data (Table 5) than its indeterminate isoline (Clark-L1). The determinate isoline also had lower color rating at both locations. But, these differences were not significant. The determinate isoline had significantly shorter plant height and lower yield.

As previously found (Pearson, 1984; Bowen, 1987), determinate cultivar Sprite, although belonging to early Maturity Group III had significantly lower LOG10CFU and color rating than Douglas, a Group IV cultivar for the first planting at both locations (Table 2). Douglas had significantly higher plant height, longer maturity and more yield than Sprite. For the second planting at Parsons, there were no significantly differences in charcoal rot infection and yield between Sprite and Douglas. Sprite had

significantly shorter plant height and earlier maturity.

No significant differences among treatments for the nodulation isolines were found in LOG10CFU (Table 6). Increasing sample size and plating more than once may reduce the experimental error and increasing precision of such experiments so that a significant difference in LOG10CFU among treatments might be revealed. The LOG10CFU combined means of the nodulated isolines tended to be higher than the unfertilized treatment of the non-nodulated isolines for both cultivars. The combined color rating value of Harosoy nodulated isoline was also higher than the unfertilized treatment of its non-nodulated isolines. The fertilized treatment of the non-nodulated isolines did not reduce LOG10CFU compared to the unfertilized treatment. But, the fertilized treatment had lower values of color rating than the unfertilized treatment at all locations for both cultivars. This difference was significant for Clark at Parsons1, Parsons2 and for the combined data.

The nodulated isolines had higher yield than the unfertilized treatment of the non-nodulated isolines at all locations for Clark and at Parsons1 and for the combined means for Harosoy (Table 6). This was significant at Parsons1 for Harosoy. The fertilized treatment of the non-nodulated isolines increased yield over the unfertilized treatment at all locations for both cultivars.

Significantly higher yield for the fertilized treatment than the unfertilized treatment occurred at Parsons1 for both cultivars and for their combined means.

Nodulation and nitrogen fertilization did not significantly change plant height and maturity when the unfertilized treatment of the non-nodulated isolines served as control (Table 6).

Greenhouse Experiment

There were no significant differences among entries for stem water content, plant height, plant height increase during reproductive growth stage and dry shoot weight (Table 7). They will not be discussed. Entry effect was significant for chlorosis scores, stem diameter, pod development days (number of days between R3 and R5), maturity, dry seed weight and color rating. LOG10CFU was unable to be obtained.

Chlorosis scores, stem diameter and pod developing days were positively correlated with color rating and negatively correlated dry seed weight, which were all significant (Table 9). There were significant correlations between chlorosis scores and stem diameter, pod developing days. Dry seed weight was negatively correlated with color rating. None of these correlation coefficients were significant when the non-nodulated isolines of the fertilized and the unfertilized were deleted from the data. Higher chlorosis scores, thicker stems, more pod developing

days, higher color rating and lower dry seed weight were mainly associated with the non-nodulated isolines (Table 8). Among these, chlorosis, thicker stems and more pod developing days might be the promoting factors for the heavier charcoal rot infection reflected by higher color rating scores.

Again, as in our field study, no significant differences were found between the determinate and indeterminate isolines of Clark (Table 8). But, the determinate isoline had lower color rating, which agreed with our field results. The determinate isoline produced higher dry seed weight. Maturities of both lines were similar. These were contradictory with the field results and might be due to the photoperiod of the early growing season in the experiment.

Among the ten entries used in this study, the determinate cultivar Sprite had the thinnest stem, least days of pod development, lowest color rating and highest dry seed weight (Table 8). The indeterminate cultivar Douglas had a significantly thicker stem, higher color rating scores and lower dry seed weight than Sprite. Douglas also had higher chlorosis scores, a longer pod developing period and later maturity than Sprite, but these differences were not significant.

The nodulated isolines had significantly lower

chlorosis scores, thinner stems, shorter pod developing days and higher seed weight than the unfertilized treatment of the non-nodulated isolines (Table 10). The nodulated isolines also had lower color rating than the unfertilized non-nodulated isolines.

Due to inadequate fertilizer used (perhaps a small portion of the 14 ppm nitrogen applied was absorbed by the soil), the difference between the fertilized and the unfertilized treatments of the non-nodulated isolines were not significant for all the traits evaluated (Table 10). The non-nodulated isolines were all poorly established. But, the fertilized treatment tended to reduce chlorosis scores, pod developing days and color rating and increase dry seed weight.

In our field and greenhouse studies with Clark near-isogenic lines, the determinate plants had non-significantly lower charcoal rot infection than the indeterminate plants. The relative yield performance of the different growth habit plants depended on environment. In the field experiments, the determinate isoline had significantly lower yield. In the greenhouse experiment, the determinate isoline had a little higher dry seed weight than the indeterminate isoline. The phenomenon that the determinate soybean plants had lower charcoal rot infection than the indeterminate soybean plants were observed by Pearson (1984) and Bowen (1987) on different

cultivars for different growth habits. We obtained the same results with Sprite and Douglas.

Bowen (1987) suggested that greater water demand during pod fill may be responsible for the increased charcoal rot infection in the indeterminate plants. But, in his hydroponic study in which adequate water were provided to all entries, the same trend occurred. Difference in the source and sink between the determinate and the indeterminate plants may be responsible for their difference in charcoal rot infection. This difference is better described as that indeterminates are source-limited and determinates sink-limited (Egli and Leggett, 1973, Hartung, 1980). A similar hypothesis on colonization by M. phaseolina has been proposed in maize (Dodd, 1977). Wyllie and Calvert (1969) demonstrated that flower removal (i.e. sink reduction) reduced the disease.

Early maturity cultivars tend to have higher colonization by M. phaseolina than late maturity cultivars (Pearson, 1984). This was also true for our field data. Pearson (1984) suggested that by flowering late, late maturity cultivars can escape the stress of hot, dry summer weather, which promotes the disease, and have less charcoal rot infection. But, in a greenhouse soil study where the environment was similar throughout the growing season, comparable results were obtained (Bowen, 1987). In our

field experiments, we observed that late planting at Parsons increased charcoal rot infection. Bristow and Wyllie (1975) also found that early planting reduced soybean charcoal rot development. It may be possible that both maturity and time of planting alter the source and sink relationship and influence charcoal rot infection. When the early maturity cultivar from the north and the late maturity cultivars from the south are grown under the same photoperiod, early maturity cultivars tend to flower early and late maturity cultivars late. Late planting soybeans are under the shortened photoperiod and flower early. For the second planting at Parsons, Sprite had much shorter plant height (source reduction) and higher yield (sink enlargement), which led to considerably higher LOG10CFU and color rating (increased charcoal rot infection) compared with the combined data from Hesston and the first planting at Parsons (Table 2).

Although nodulation tended to increase soybean seed yield in both field and greenhouse studies, the effects of nodulation on charcoal rot infection were inconsistent. The soil fertility on which to conduct the studies may influence charcoal rot infection more on the unfertilized non-nodulated isolines as control compared with what it does on the nodulated isolines. Nodulation may increase charcoal rot infection when the study is conducted in the field with balanced fertility and the control has low

charcoal rot infection. This might be the case for our field study. Nodulation may reduce charcoal rot infection when the study is conducted on poor soil and the control has high charcoal rot infection. This might be true for the greenhouse study.

Nitrogen fertilization reduced charcoal rot infection when charcoal rot was measured by color rating and increased seed yield in both field and greenhouse studies. Under nutrient-deficient conditions, plants may have less vigorously vegetative growth and weakened defense mechanisms. This may make plants prone to charcoal rot. Nitrogen fertilization on the soil that lacks nitrogen certainly improves plant vegetative vigor and health, decreases charcoal rot infection and increases seed yield. Higher level of nitrogenous fertilizer have been reported to increase the severity of many diseases caused by soil borne pathogens (Henis and Katan, 1975). This may not be the case for soybean charcoal rot.

Table 1. Analysis of variance for charcoal rot data and major agronomic traits from field study.

Source of variation	Degree of freedom	Mean squares[+]				
		LOG10CFU	Color rating	Plant height	Maturity	Yield
ENV	2	14.8*	11.6	195**	617**	164322
REP(ENV)	5	2.2**	2.3	1	0	43033
ENTRY	9	2.3**	24.7**	460**	308**	2688720**
ENV*ENTRY	15	1.0*	8.6**	33**	13	153660
ERROR	39	0.4	1.0	8	7	90669

*,** Significant at 0.05 and 0.01 probability levels, respectively.
+ Based on data from all three environments.

Table 2. Charcoal rot data and major agronomic traits from field study.

Cultivar	LOG10CFU		Color rating		Yield		Plant height		Maturity	
	1#	2$	1	2	1	2	1	2	1	2
	per gram		0-10		kg/ha		cm		days+	
Harosoy										
Nod	3.5	-	9.3	-	891	-	76	-	98	-
Non-nod,Unf	3.3	-	8.6	-	738	-	77	-	100	-
Non-nod,Fer	3.2	-	8.4	-	1108	-	73	-	100	-
Sprite	0.9	3.9	1.4	6.0	1861	2014	46	25	115	105
Williams 82	2.7	3.1	5.3	4.6	2407	2670	85	86	121	113
Clark										
Det,Nod	-	3.7	-	5.5	819	614	32	19	113	111
Indet,Nod	2.1	3.6	5.6	7.2	1752	2189	78	85	120	110
Non-nod,Unf	2.0	3.5	5.8	6.9	1480	1829	77	83	118	109
Non-nod,Fer	2.2	3.0	4.6	5.1	2085	2297	79	84	119	113
Douglas	2.0	3.5	5.1	4.3	2464	2269	87	81	125	116
LSD(0.05)	1.0	0.9	1.2	2.1	448	306	8	10	4	5

+ From planting to maturity.
# Combined first planting data at Hesston and Parsons.
$ Second planting data at Parsons.

Table 3. Comparison of charcoal rot data and agronomic traits from field study among three environments.

Environment[+]	Planting Date	LOG10CFU	Color rating	Yield	Plant height	Maturity
		per gram	0-10	kg/ha	cm	days[#]
Hesston	May 10	1.9a*	3.3a	2058ab	68a	-
Parsons1	May 22	2.1a	6.0b	1959a	83b	120a
Parsons2	June 10	3.5b	5.7b	2211b	74a	111b

* Means within a column followed by the same letter are not significantly different at 0.05 probability level according to LSD procedure.
+ Entries used are Sprite, Williams 82, Clark-L1, L63-1889 (fertilized), L63-1889 (unfertilized) and Douglas, each of which was present at all the three environments.
# From planting to maturity.

Table 4. Pearson correlation coefficients among charcoal rot data and major agronomic traits from field study at Hesston and Parsons1 based on entry means (n=10).

	LOG10CFU	Color rating	Plant height	Maturity
	r			
Yield	-0.61	-0.73*	0.42	0.84**
LOG10CFU		0.95**	0.52	-0.71*
Color rating			0.48	-0.74*
Plant height				0.13

*, ** Significant at 0.05 and 0.01 probability levels, respectively.

Table 5. Comparison of charcoal rot data and major agronomic traits between determinate and indeterminate isolines of Clark in field study.

Environ	Growth habit	LOG10CFU	Color rating	Yield	Plant height	Maturity
		per gram	0-10	kg/ha	cm	days
Hesston	Det	1.5a*	4.2a	978a	34a	-
	Indet	2.0a	4.6a	1792a	66b	-
Parson1	Det	3.7a	5.5a	614a	19a	111a
	Indet	3.6a	7.2a	2189b	85b	110a
Combined	Det	2.6a	4.8a	796a	26a	111a
	Indet	2.8a	5.9a	1991b	75b	110a

* Means within a column followed by the same letter are not significantly different at 0.05 probability level according to LSD procedure.

Table 6. Charcoal rot data and major agronomic traits from field study among nodulation isolines.

Variable	Treatment	Clark				Harosoy		
		Hess	Par1	Par2	Combined	Hess	Par1	Combined
LOG10CFU per gram	Nod	2.0a*	2.2a	3.6a	2.6a	3.5a	3.4a	3.5a
	Unfert	1.3a	2.8a	3.5a	2.5a	3.7a	2.8a	3.3a
	Fert	1.9a	2.5a	3.0a	2.5a	3.6a	3.0a	3.3a
Color rating 0-10	Nod	4.6a	6.7ab	7.2a	6.2a	9.7a	8.9a	9.3a
	Unfert	3.1a	8.5a	6.9a	6.2a	10.0a	7.3ab	8.6a
	Fert	2.7a	6.4b	5.1b	4.8b	9.9a	6.9b	8.4a
Plant height cm	Nod	67a	91a	84a	81a	66a	85a	76a
	Unfert	66a	91a	85a	81a	69a	87a	78a
	Fert	65a	86a	83a	78a	74a	81a	77a
Maturity days+	Nod	-	119a	113a	116a	-	100a	100a
	Unfert	-	120a	110ab	115ab	-	98a	98a
	Fert	-	118a	109b	113b	-	100a	100a
Yield kg/ha	Fert	2086a	2084a	2297a	2156a	684a	1533a	1108a
	Nod	1792a	1713ab	2189a	1898ab	554a	1229b	891b
	Unfert	1564a	1396b	1829a	1596b	619a	856c	738b

* Means within a column followed by the same letter are not significantly different at 0.05 probability level according to LSD procedure.
+ From planting to maturity.

Table 7. Analysis of variance for charcoal rot and agronomic traits in greenhouse study.

Source of variation	Degree of freedom	Mean squares				
		Chlorosis scores	Stem diameter	Stem water content	Plant height	Plant height increase+
REP	3	0.7	0.003	207	0	1.2
ENTRY	9	4.8**	0.010*	146	29	2.4
ERROR	27	0.4	0.003	71	13	1.4
		Pod developing days	Maturity	Dry shoot weight	Dry seed weight	Color rating
REP	3	0.7	42	0.2	0.3	1.5
ENTRY	9	11.0*	64**	0.2	2.9**	5.4**
ERROR	27	3.9	17	0.3	0.3	1.1

*, ** Significant at 0.05 and 0.01 probability levels, respectively.
+ During reproductive stage (R1-R7).

Table 8. Charcoal rot data and agronomic traits of greenhouse study.

Cultivar	Chlorosis scores	Stem diameter	Pod developing days	Maturity	Dry seed weight	Color rating
	0-3	cm	R3-R5	days+	g	0-5
Harosoy						
Nod	0.5	0.535	6.8	124	2.3	2.9
Non-nod, Unf	2.3	0.578	9.1	127	0.8	3.8
Non-nod, Fer	1.5	0.640	6.0	131	1.4	3.6
Sprite	0	0.498	4.5	133	2.7	0.6
Williams 82	0	0.570	4.5	136	2.2	1.1
Clark						
Det, nod	0	0.595	6.5	136	1.3	2.3
Indet, Nod	0	0.577	5.8	137	1.1	2.9
Non-nod, Unf	2.5	0.635	9.4	136	0.4	4.4
Non-nod, Fer	2.5	0.680	8.0	134	0.1	3.1
Douglas	0.5	0.625	4.8	136	1.5	3.4
LSD (0.05)	0.9	0.079	2.9	6	0.8	1.5

+ From planting to maturity.

Table 9. Pearson correlation coefficients among agronomic traits and charcoal rot data in greenhouse study based on entry means (n=10).

	Stem diameter	Pod developing days	Maturity	Dry seed weight	Color rating
			r		
Chlorosis scores	0.64*	0.85**	-0.22	-0.78**	0.72*
Stem diameter		0.42	0.33	-0.82**	0.65*
Pod developing days			-.30	-0.76*	0.73*
Maturity				-0.23	-0.17
Dry seed weight					-0.74*

*, ** Significant at 0.05 and 0.01 probability levels, respectively.

Table 10. Charcoal rot data and agronomic traits in greenhouse study for nodulation isolines.

Treatment	Chlorosis score	Stem diameter	Pod developing days	Maturity	Dry seed weight	Color rating
	0-3	cm	R3-R5	days+	g	0-5
Nodulated	0.3a*	0.557a	6a	131a	1.7a	2.9a
Fertilized	2.0b	0.657b	7ab	132a	0.8b	3.3a
Unfertilized	2.4b	0.611b	9b	131a	0.6b	4.1a

* Means within a column followed by the same letter are not significantly different at 0.05 probability level according to LSD procedure.
+ From planting to maturity.

PART II IN VITRO RESPONSE OF SOYBEAN CULTIVARS TO THE TOXIN OF *M. PHASEOLINA*

ABSTRACT

In vitro selection of resistance has been studied in many crop species. Recent development of tissue culture techniques in soybeans made it possible to make an attempt of in vitro selection of resistance to the toxin of *M. phaseolina*, causal fungus of soybean charcoal rot. In this study, the variation of callus response to the toxin among selected cultivars were evaluated. Four soybean cultivars were used. Douglas and Sprite are the highest and lowest infection cultivars, respectively, under the field conditions. Callus initiation was on MS medium with 5 gm/L 2,4-D. The toxin was furnished by the dialyzed culture filtrate. Callus response was characterized by growth rate reduction and browning scores. Bay was more susceptibility than all other cultivars with respect of growth reduction and browning scores. Douglas seemed to be more susceptible than Sprite and Williams 82, which agreed with the results of field root colonization studies. Since the toxin may play a very important role in the diseasing process, in vitro selection of resistance to the toxin may prove to be a feasible approach to utilize the in vitro variation in reducing the damage of this disease.

Charcoal rot is an important fungal disease for soybeans in Kansas as well as in the midwest United States and the world. Resistant cultivars have been employed to control many crop diseases. However, resistance to charcoal rot in soybeans has not been identified. But, variation of response to M. phaseolina among cultivars exists (Agarwal and Sarbhoy, 1976; Pearson et al., 1984). The finding of resistance depends upon successful screening techniques. Such techniques have not been developed for charcoal rot in soybeans. Resistance may prove to be a useful method solving charcoal rot problem.

One of the selection techniques that has been most popular in the last few years is in vitro selection. Moving to an alien environment may make plant tissues and cells display more genetic irregularity and gene mutations (Brettell and Ingram, 1979). It may be possible to make use of this genetic heterogeneity. Repeated exposure of pieces of callus from either susceptible genotypes or resistant genotypes to increasing concentrations up to sublethal concentrations of toxin, culture filtrate or even the pathogen can increase selection pressure and favor those mutations of higher resistance. Selection and regeneration into plants of the fast-growing section of the callus on the medium containing toxin, filtrate or pathogen may produce resistant cultivars. This work has been done with several plant species. Gengenbach et al. (1977) cultured

immature embryos from Texas male-sterile cytoplasm corn genotype BC1A188(T) which was susceptible to Helminthosporium maydis race T and its toxin. After four selection cycles in the presence of progressively higher concentrations of the toxin, culture calli were regenerated into plants. These plants were resistant to the toxin. Selection from protoplast-derived calli of tobacco resistant to the toxins from Pseudomonas syringae pv tabaci, which causes wildfire disease, and from Alternaria alternata pathotype tobacco, which causes brown spot, yielded resistant regenerates (Thanutong et al., 1983). An assay of the R1 generation indicated that the resistance shown by R0 plants against both diseases was heritable. Latunde-Dada (1983) regenerated two types of plants from alfalfa mesophyll protoplasts: normal type and variants. The study showed that the variants which were of high polyploidy were highly tolerant to the wilt fungus Verticillium albo-atrum. Behnke (1979) also reported the regeneration of potato plants rrom calli which were resistant to the culture filtrate of Phytophthora infestans.

Previous research has indicated that resistance to pathogen, pathotoxin or culture filtrate containing pathotoxin at the whole plant level will be expressed in culture (Deaton et al., 1982; Gengenbach et al., 1977; Gray

et al., 1986; Hartman et al., 1984; Helgeson et al., 1976) and resistance will not be lost when resistant calli are regenerated into plants (Behnke, 1979; Gengenbach et al., 1977; Hartman et al., 1984; Thanutong et al., 1983).

Recent developments of tissue culture techniques in soybeans make possible an attempt to select for resistance to the toxin of M. phaseolina, pathogen of soybean charcoal rot. Since Christianson et al. (1983) first obtained plant regeneration from cultures from one immature embryo of one genotype in 1983, several laboratories have independently reported success in regenerating plants from soybean callus cultures. Regeneration of fertile plants from immature embryos has become routine (Barwale et al., 1986; Ghazi, 1986; Ranch et al., 1985). Regeneration of plantlets from soybean protoplasts was also obtained (Wei et al, 1988).

Exploring the possible application of in vitro selection of resistance to the toxin of M. phaseolina as the general purpose, this study was conducted to evaluate the variation of callus response to the toxin of M. phaseolina among selected soybean cultivars.

MATERIALS AND METHODS

Soybean Callus Production

Four soybean cultivars, Bay, Douglas, Sprite and Williams 82 were used in this study. Sprite and Douglas have been shown to be lowest and highest infection cultivars by M. phaseolina, respectively, in the field studies (Pearson et al., 1984; Pearson, 1987; Bowen, 1987). The plants were grown in the greenhouse. The conditions were as described in the first part of this paper. Ten plants in ten pots for each cultivar were planted at one month intervals. The pots contained a 1:1 mixture of peat and soil covered by a thin layer of sand. They were watered daily.

Soybean pods which had embryos 4-6 mm long were removed from the plants. The pods were surface sterilized first in a solution of 0.525% sodium hypochlorite (made from 10% Clorox, a commercial bleach containing 5.25% of sodium hypochlorite) with 0.05% Nonidet P-40 (Octylphenoxypolyethoxyethanol) for 10-15 minutes. They were then rinsed twice with sterile distilled water. The pods were opened with a scalpel and a pair of tweezers under an AO580 scope. Immature embryos were taken out and dissected to remove radicals. Cotyledonary pieces were placed, with adaxial side down, in 100x15 mm petri dishes containing 20 ml of culture medium. The whole process was

done aseptically in a laminar flow hood.

The tissue culture medium was MS medium (Murashige and Skoog, 1962) with 5 mg/l of 2,4-D (2,4-dichlorophenoxyacetic acid), which supported the callus growth (Ranch et al., 1985). The medium had 3% sucrose and was semi-solidified by adding 0.7% Difco purified agar. The pH was adjusted to 5.7 before the medium was autoclaved at 121 ^{o}C for 20 minutes.

Cultures were incubated in a Percival 1-35LLVL incubator for callus initiation and multiplication. The incubator temperature was maintained at 28^{o}C. Photoperiod was 12 hours with illumination supplied by General Electric Cool-white fluorescent tubes (60 umol m^{-2} s^{-1}). All callus cultures were transferred to the same fresh medium at 20 day intervals.

Culture Filtrate Preparation

The toxin of M. phaseolina was furnished by the dialyzed culture filtrate. Isolate 87PAR01, used in this study, was isolated from the roots of Harosoy at Parsons in 1987. The fungus isolation procedure used PDA (Potato Dextrose Agar) medium. The root tissues were first surface sterilized in 15% Clorox solution followed by washing with sterile distilled water. The tissues were transferred regularly until a pure culture of M. phaseolina was obtained. The culture was maintained in the dark at 30 ^{o}C.

The isolate was grown in soybean seed (Douglas)

extract broth with 0.5% carboxymethylcellulose and 0.5% pectin (Sigma Chemical Co., St. Louis, MO) in the dark at 30 ^{o}C as described by Dhingra and Sinclair (1974). After 15 day incubation, the culture was first filtered through cheesecloth and then through Whatman No. 42 filter paper. One liter of crude culture filtrate was concentrated to 60 ml in rotary-evaporator at 60 ^{o}C. This material was dialyzed against 4 L distilled water for 72 hours at 4 ^{o}C with constant stirring to remove salts. The water was changed daily. The dialysate was collected, reduced to the desirable amount (350 ml in the first run and 200 ml in the second) and filtered through Whatman No. 42 filter paper again. The dialyzed culture filtrate (referred as culture filtrate) was used in this study.

Incorporation of Culture Filtrate into Tissue Culture Medium and Quantification of Callus Response

The final volume of the tissue culture medium before autoclaving was adjusted to accommodate the culture filtrate so that the concentration of the tissue culture medium was not changed. The pH of culture filtrate was adjusted to 5.7, the same as that of tissue culture medium. The culture filtrate was filter-sterilized through a 0.45-um Millipore filter, and incorporated into tissue culture medium. The medium was pipetted into 100x15 petri dishes with 20 ml per plate. It was usually seasoned for 1 day

before use. Two runs were conducted. In the first run, there were 5 concentrations from 0, as control, to 50% and 3 replications. For 4 cultivars, there were totally 60 plates. In the second run, there were 2 concentrations, 0 and 25%, 3 dates at which the ending weights were obtained and 3 replications. For 4 cultivars, there were totally 72 plates. In the first run, there were 5 calli (0.66 to 1.35 g totally) in each plate. In the second run, there were 4 calli (0.59 to 0.89 g totally) in each plate. Different replications were established by putting all plates of one replication into a plastic box or on different layers in the incubator. The plates were incubated at the same conditions described earlier for callus production.

The beginning and ending weights of calli in each plate was aseptically determined on a balance in the laminar flow hood for each experiment. The average daily growth rate was calculated using the formula below:

$$GR=\left(\sqrt[d]{\frac{EW}{BW}}-1\right)\times 100$$

GR: average daily growth rate of each plate over the incubation period.

EW: ending weight of calli in each plate

BW: beginning weight of calli in each plate

d: days of incubation

Browning scores were also determined in the first run.

Complete browning on the whole callus surface was rated 5, partial browning 2.5, and no browning 0.

RESULTS AND DISCUSSION

Callus Growth

When cotyledonary pieces from immature embryos were placed on MS medium with 5 mg 2,4-D, callus was initiated within a month. When it was transferred to the same fresh medium, it multiplied. Good callus showed a fresh, creamish color. Regular transfer of the callus at 15-20 day intervals gave the best callus growth. If the callus was not transferred to the new medium in a month, it turned green and was not usable in this study.

Callus growth was very uneven. The highest daily growth rate for the first 15 day incubation was 12.2%. The lowest was only 1.1%. Even within one plate, one callus might grow very fast, another very little. The normal average daily growth rate was around 8%.

Callus grew faster for the first few days after it was transferred to fresh medium. For example, in the second run, the daily growth averaged 7.5% for the first 5 days on new medium, 4.9% for the first 10 days and 4.5% for first 15 days. If there was a log phase, it must have been reached very fast. After 5 days on the same medium, the callus were growing slowly.

Differential callus growth among cultivars was noticed. In the first run, the control daily growth rate of Douglas for the first 15 day incubation was 9.4% and that of Bay

was 5.0%. In the second run, that of Douglas was 7.3% and that of Bay was 4.5%. The daily growth rates of Sprite and Williams 82 were 8.2% (first run) and 7.3% (second run), respectively. Bay had significantly lower daily callus growth rate than other cultivars.

Callus Response to Culture Filtrate

In the first run presented in Table 1, regression analysis using the concentration of culture filtrate as independent variable and the growth rate reduction (a percentage of the control growth rate) dependent variable indicated $y=-0.067x+0.865$ with $R^2=0.443$. Higher concentration of culture filtrate was accompanied by more growth rate reduction. The growth rate at 12.5% concentration was significantly lower than that of control.

Browning was another phenomenon observed when callus was placed on medium containing toxin. Severe browning usually occurred on the surface of callus. Weeks (1987) suspected that cells on the surface of callus were more recent and divided quickly. These cells might be actively involved in transporting toxin. Therefore, browning first occurred in these cells.

For some calli, browning was on the whole surface. For others, browning only occurred on part of their surface. There was also no browning for still others. When browning occurred on the whole surface in some calli, they might loose water and became smaller in size. Sometimes, new

sections grew out of the browning calli. They might grow into new calli. These might be derived from resistant cells and serve as the basis of in vitro selection.

Browning scores were increased by the higher concentration of culture filtrate applied (Table 1). Regression analysis using the concentration of culture filtrate as independent variable and browning scores as dependent variable indicated $y=0.165x+0.568$ with $R^2=0.126$. The browning scores at 12.5% concentration were significantly higher than that of control.

Differential Callus Response to Culture Filtrate among Cultivars

Correlation analysis indicated that there was a negative correlation between the control growth rate and the growth rate reduction of treatments. In the first run, $r=-0.19$, which was not significant at 0.05 probability level. In the second run, $r=-0.39$, which was significant at 0.05 probability level. That means when the callus of a cultivar grew faster, i.e. high in its control growth rate, it tended to have more growth rate reduction, i.e. low in its percentage of the control growth rate when subjected to culture filtrate. When a cultivar whose callus grows faster is evaluated for in vitro resistance to the toxin, it may show more susceptibility than others with slow-growing callus if the growth rate change is used as the sole mean

of selection. But, in situ, a soybean cultivar whose callus grows faster may not necessarily be more susceptible to the toxin of M. phaseolina

It is suspected that the growth rate reduction which increased with increasing concentration of culture filtrate might not solely result from the effect of toxin. The tissue culture medium was derived from many experiments and modifications and was a balanced, refined medium. Addition of exotic substances might affect such a balance. This effect might be proportional to the amount of the substance added. This might be true for the second run. The whole experiment average growth rate reduction of the first run was 53% of control. That of the second run was only 75%.

In the second run as discussed earlier, there was a significant negative correlation between the control growth rate and growth rate reduction expressed as the percentages of the control growth rate. The overall growth rate reduction of the experiment was also lower. No intense browning occurred. Analysis indicated that no significant differences occurred among cultivars and culture filtrate concentrations. Therefore, the results of the culture filtrate effect in the second run are not presented. Only the results of the first run are discussed here.

Williams 82 had less growth rate reduction at all concentrations and significantly less at 12.5%, 50%

concentrations and also for the overall average than all other cultivars (Fig. 1). No intense browning was observed for this cultivar (Fig. 2). Williams 82 may be an in vitro resistant cultivar to the toxin. Part of the response might be attributed to the low control growth rate of Williams 82 (4.3%) (Fig. 1). Its control growth rate in the second run was 7.3%.

In spite of its low control growth rate, Bay had more growth rate reduction than all other cultivars at 25%, 37.5% culture filtrate concentrations and for overall means (Fig. 1). The difference of Bay was significant at 0.05 probability level from Williams 82 at all culture filtrate concentrations and from Douglas at 25.5% and 37.5% concentrations. But, the growth rate reduction of Bay was not significantly different from Sprite at all concentrations and Douglas at 12.5% and 50% concentrations. Bay had significantly higher browning scores at 12.5%, 25.5% and 37.5% concentrations and significantly higher overall means than all cultivars (Fig. 2). But, it was not significantly different from Douglas and Sprite at 50.0% concentration.

Douglas had more growth rate reduction at 12.5% concentration than at either 25.0% or 37.5% concentrations. At the latter two concentrations, the results might be due to the fast growing calli from the

browning calli. Douglas had higher browning scores at 37.5% and 50.0% concentrations and significantly higher browning scores at 12.5% and 25.0% concentrations than Sprite. If the response of Douglas with respect of growth rate reduction at 12.5% concentration was its true response, Douglas was more susceptible than Sprite in the callus response to the toxin.

Sprite and Williams 82 showed less charcoal rot infection than Douglas in the previous and our field (first planting) and greenhouse studies. The results of our callus study obtained on the three cultivars seemed to agree with the above field results. However, the relationship between the degree of colonization by the fungus in the field and the callus response to the toxin may be more complex than this. The most susceptible cultivar Bay in this study has not been reported to be the high infection cultivar in field studies (Pearson, 1984; Bowen, 1987). The degree of colonization by the fungus may be influenced by many external factors of environment and internal factors of host and parasite physiology, for example maturity and may not be directly controlled by genetic information.

Toxicity Determination of Culture Filtrate

Since the molecular weight of this toxin is unknown, the amount of toxin added to tissue culture medium could not be quantified. This causes difficulty in comparing results of different experiments. Some bacteria were

reported to be sensitive to the toxin (Pearson, 1982). We tried to use a bioassay technique with bacteria to determine the toxicity of culture filtrate (Pearson, 1982). One day old culture of bacteria was transferred to Luria broth and grown to a log phase. Then the bacteria suspension was mixed with Luria soft agar medium (0.6% agar) in a 1:2 ratio. One ml of the Luria soft agar medium containing bacteria was pipetted into a petri dish containing Luria hard agar medium (1.5% agar) to form a bacteria lawn. The plates were incubated for 4 hours. Meanwhile, dilutions up to 1/256 of culture filtrate were made. When the bacteria were ready, a 10 ul drop of each dilution of culture filtrate was spotted on the bacteria lawn of each plate. They were incubated for 14 hours. The inhibition zone in each drop of culture filtrate were examined. The lowest dilution that expressed inhibition was determined and used for the culture filtrate toxicity. Three bacteria, Pseudomonas phaseolicola (82-H1), Erwinia amylovora (EA 101) and Serratia marcesens from Plant Pathology, Kansas State University were used. But, the results were ideal. Sometimes, mixed inhibition made it difficult to identify the lowest inhibitory dilution. Sometimes, no inhibition occurred. The problem might be the bacteria growth stage. Sensitivity of bacteria to the toxin is also very important.

Problems Associated with this Study

Callus was an important factor in the success of such a study. Tissues from spring or summer-planting plants seemed to give creamish, fresh, and normal growth callus. Only this kind of callus may give the real response of its cultivar. Smaller callus grew slowly and responded later. Large calli often had new sections growing out of them which confounded the effect of toxin. The usual size used in this study was 0.20 g. Another difficulty in this study was synchronization of the callus multiplication and the culture filtrate preparation. the culture filtrate preparation required 20 days. At about 5 days after the fungus was started in liquid culture, callus should be ready and transferred to fresh medium.

Early in this study, the partially purified toxin was used. Only 8 ml of it was produced from each 2 L fungus culture. It was hard to do an experiment with 8 ml of the partially purified toxin which could be be statistically analyzed. It was also time-consuming. Therefore, the dialyzed culture filtrate was used later on.

Most failures came from the toxicity of culture filtrate. The ability of the fungus to produce toxin seemed to be weakened as the fungus was maintained longer on PDA medium. The most successful experiment in this study used a newly-isolated culture of the fungus. In future experiments, it may be helpful to keep root samples,

isolate the fungus when needed, not use the culture for a long period.

The toxin may have a very important role for the fungus. Reddish-brown lesions on either soybean seedlings or older plants may be the result of the toxin action. The toxin caused browning in soybean callus and may also be responsible for the browning occurring on the stem and shoot cortices, petioles, leaves, pods and the pithy and vascular tissues. The fungus may be a necrotrophic parasite (Wood, 1967) and use the toxin to kill the tissues before its colonization by sclerotia. This is supported by our experimental evidence. In trying to find the variation of callus response to M. phaseolina, we did experiments in which callus was incorporated alive into water agar or tissue culture medium above which there was a layer of PDA medium which had a plug of the fungus on it. We found that although mycelium grew vigorously on the top for a fairly long period, the callus remained fresh color and alive until sclerotia formed. This indicated that something must be secreted to kill the tissues before sclerotial colonization. This might be the toxin.

In this study, we found that there was a variation among cultivars in terms of callus response to the toxin. Low infection cultivars also showed certain in vitro resistance to the toxin. In vitro selection to the toxin

may prove to be a feasible approach to reduce soybean charcoal rot damage.

Table 1. Effect of culture filtrate on the callus daily growth rate and browning scores for the first 15 days[+].

Concentration	Growth rate reduction (of control)	Browning scores (0-5)
0%	100%a*	0.18a
12.5%	60%b	1.33b
25.0%	41%c	1.67b
37.5%	35%c	1.75b
50.0%	25%c	2.04b

* Means within each column followed by the same letter are not significantly different at 0.05 probability level according LSD procedure.

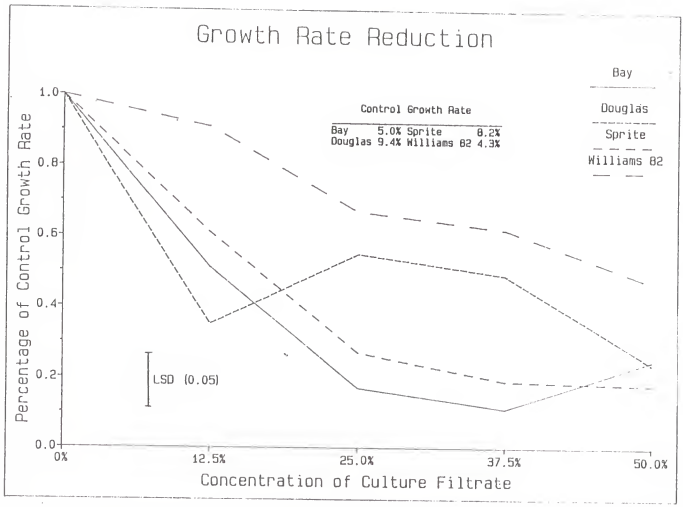

Fig. 1. Differential cultivar response to culture filtrate of M. phaseolina with respect of callus growth rate reduction.

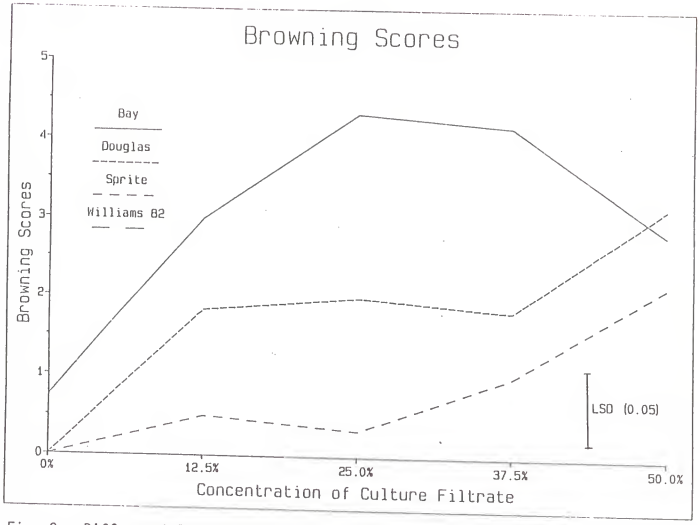

Fig. 2. Differential cultivar response to culture filtrate of M. phaseolina with respect of callus browning (note that Williams 82 is hidden by X-axis).

1. Agarwal, D. K., and A. K. Sarbhoy. 1976. Evaluation of soybean germplasm for resistance against Macrophomina phaseolina. Indian Phytopathology 29:190-191.
2. Athow, K. L. 1973. Fungal Diseases, p477. In Soybean, Production, and Uses. B. E. Caldwell (ed.). American Society of Agronomy, Inc., Publisher. Madison, Wisconsin.
3. Barwale, U. B., H. R. Kerns, and J. M. Widholm. 1986. Plant regeneration from callus cultures of several genotypes via embryogenesis and organogenesis. Planta 167:473-481.
4. Behnke, M. 1979. Selection of potato callus for resistance to culture filtrates of Phytophthora infestans and regeneration of resistant plants. Theor. Appl. Genet. 55: 69-71.
5. Bowen, C. R. 1987. The Effect of Blends, Rate of Planting, and Growth Habit on Charcoal Rot Infection and Yield. A Ph. D. Dissertation. Kansas State University. Manhattan, Kansas.
6. Brettell, R. I. S., and D. S. Ingram. 1979. Tissue culture in the production of novel disease-resistant crop plants. In Biol. Rev. 54:329-345.
7. Bristow, P. R., and T. D. Wyllie. 1975. The effect of crop rotation and date of planting on charcoal rot of soybean. Proceedings of the American Phytopathological Society 2:83 (abstract).
8. Bristow, P. R., and T. D. Wyllie. 1986. Macrophomina phaseolina, another cause of the twin-stem abnormality disease of soybean. Plant Disease 70 (12):1152-1153.
9. Canaday, C. H., and A. F. Schmitthenner. 1979. The effect of nitrogen on Phytophthora root rot of soybean (Abstr.). Phytopathology 69:539.
10. Chan, Y. H., and W. E. Sackston. 1969. Mechanism of pathogenesis in Sclerotium bataticola on sunflowers. 1. Production and translocation of a necrosis-inducing toxin. Can. J. Bot. 47:1147-1151.
11. Chan, Y. H., and W. E. Sackston. 1973. Nonspecificity of the necrosis-inducing toxin of Sclerotium batalicola. Can. J. Bot. 51:690-692.
12. Christianson, M. L., D. A. Warnick, and P. S. Carlson. 1983. A morphogenetically competent soybean suspension culture. Science 222:632-634.
13. Deaton, W. R., G. J. Keyes, and G. B. Collin. 1982. Expressed resistance to black shank among tobacco callus cultures. Theor. Appl. Gent. 63:65-70.
14. Dhingra, O. D., and J. B. Sinclair. 1973. Variation among isolates of M. phaseoli (Rhizoctonia bataticola) from different regions. Phytopath. Z. 76:200-204.

15. Dhingra, O. D., and J. B. Sinclair. 1974. Isolation and partial purification of a phytotoxin produced by Macrophomina phaseolina. Phytopath. Z. 80:35-40.
16. Dhingra, O. D., and J. B. Sinclair. 1978. Biology and Pathology of M. phaseolina. Unversidade Federal De Vicosa, Brazil.
17. Dodd, L. 1977. A photosynthesis stress-translocation balance concept of corn stalk rot. 32nd Annual Corn and Sorghum Conference 32:112-130.
18. Egli, D. B. and J. E. Leggett. 1973. Dry matter accumulation patterns in determinate and indeterminate soybeans. Agron. J. 13:220-222.
19. Gengenbach, B. G., C. E. Green, and C. M. Donovan. 1977. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proc. Natl. Acad. Sci. USA 74:5113-5117.
20. Ghazi, T. D., H. V. Cheema, and M. W. Nabors. 1986. Somatic embryogenesis and plant regeneration from embryogenic callus of soybean, Glycine max L. Plant Cell Report 5: 452-456.
21. Gray, L. E., Y. Q. Guan, and J. M. Widholm. 1986. Reaction of soybean callus to culture filtrates of Phialophora gregata. Plant Sci. 47:45-55.
22. Hartman, C. L., T. J. McCoy, and T. R. Knous. 1984. Selection of alfalfa (Medicago sativa) cell lines and regeneration of plants resistant to the toxin(s) produced by Fusarium oxysporum f. sp. Medicaginis. Plant Sci. Lett. 34: 183-194.
23. Henis, Y., and Katan, J. 1975. Effect of inorganic amendments and soil reaction on soilborne plant diseases. Pages 100-106 in: Biology and Control of Soil-Borne Plant Pathogens. G. W. Bruehl, ed. Am. Phytopathol. Soc., St. Paul, MN 216pp.
24. Hartung, R. C., J. E. Specht, and J. H. Williams. 1980. Agronomic performance of selected soybean morphological variants in irrigation culture with two row spacings. Crop Sci. 200:604-609.
25. Ilyas, M. B., M. A. Ellis, and J. B. Sinclair. 1976. Effect of soil fungicides on M. phaseolina variability in soil and in soybean stem pieces. Phytopathology 66:355-359.
26. Latunde-Dada, A. O., and J. A. Lucas. 1983. Somaclonal variation and reaction to Verticillium wilt in Medicago sativa L. plants regenerated from protoplasts. Plant Sci. Lett. 32:205-211.
27. Meyer, W. A., J. B. Sinclair, and M. N. Khare. 1973. Biology of Macrophomina phaseoli in soil studied with selective media. Phytopathology 63:613-620.
28. Meyer, W. A., J. B. Sinclair, and M. N. Khare. 1974. Factors affecting charcoal rot of soybean seedlings. Phytopathology 64:845-849.

29. Pearson, C. A. S. 1982. Macrophomina phaseolina, causal organism of charcoal rot of soybean. A Master's Thesis. Kansas State University.
30. Pearson, C. A. S., F. W. Schwenk, and F. J. Crowe. 1984. Colonization of soybean roots by Macrophomina phaseolina. Plant Disease 68 (12):1086-1088.
31. Pearson, C. A. S., J. F. Leslie, and F. W. Schwenk. 1986. Variable chlorate resistance in M. phaseolina from corn, soybean, and soil. Phytopathology 76:646-649.
32. Pearson, C. A. S. 1987. Field and Laboratory Observations on Host-parasite Relationships of Soybean with M. phaseolina. A Ph. D. Dissertation. Kansas State University. Manhattan, Kansas.
33. Ranch, J. P., L. Oglesby, and A. C. Zielinski. 1985. Plant regeneration from embryo-derived tissue cultures of soybeans. In Vitro Cellular & Developmental Biology 21 (11):653-658.
34. Sinclair, J. B. 1983. Root and stalk rots caused by Macrophomina phaseolina in legumes and other crops. In Sorghum Root and Stalk Rots -- A Critical Review. Proceedings of the Consultative Group Discussion on Research Needs and Strategies for Control of Sorghum Root and Stalk Rot Diseases. Bellagio, Italy. 27 Nov.--2 Dec. 1983.
35. Sinclair, J. B. (ed.). 1984. Compendium of Soybean Diseases. 2nd edition. American Phytopathological Society, St. Paul, MN.
36. Thanutong, P., I. Furusawa, and M. Yamamoto. 1983. Resistant tobacco plants from protoplast-derived calluses selected for their resistance to Pseudomonas and Alternaria toxins. Theor. Appl. Genet. 66:209-215.
37. Weeks, J. T. 1987. Tissue Culture in Soybean Improvement. A M.S. Thesis. Kansas State University. Manhattan, Kansas.
38. Wei, Z. M., and Z. H. Xu. 1988. Regeneration of plantlets from soybean protoplasts. Plant Physiology Communications. (2):53-54.
39. Wood, R. K. S. 1967. Physiological Plant Pathology. Blackwell Scientific Publication Ltd. Oxford, United Kingdom. 570pp.
40. Wyllie, T. D., and O. H. Calvert. 1969. Effect of flower removal and pod set on formation of sclerotia and infection of Glycine max by Macrophomina phaseoli. Phytopath. 59:1243-1245.

INFLUENCE OF GROWTH HABIT, NODULATION, AND NITROGEN FERTILIZATION ON THE INCIDENCE OF SOYBEAN CHARCOAL ROT AND IN VITRO RESPONSE OF SOYBEAN CULTIVARS TO THE TOXIN OF *M. PHASEOLINA*, CAUSAL FUNGUS OF THE DISEASE

by

HONGRUN YU

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ABSTRACT

Charcoal rot is an important fungal disease for soybeans in Kansas as well as in the mid-west United States and the world. Cultivars of different growth habits were reported to have differential infections. Previous study showed that isolates of M. phaseolina, causal agent of the disease, from soybean and corn, another host, responded differently to chlorate, an analog of nitrate. Because soybean isolates were chlorate-sensitive, they might be also sensitive to intermediates of nitrate metabolism. Both field and greenhouse studies were conducted to investigate the influence of growth habit, nodulation and nitrogen fertilization on the non-nodulated isolines on the root colonization by the chlorate-sensitive fungus in soybeans with growth habit and nodulation near-isogenic isolines of Clark and Harosoy. There was no significant difference between the determinate and indeterminate isolines in charcoal rot infection measured by LOG10CFU and color rating. But, the determinate plants tended to have less LOG10CFU and color rating. Nitrogen reduced the disease when it was measured by color rating and increased seed yield on the non-nodulated isolines. Although nodulation increased seed yield compared with the unfertilized non-nodulated isolines, its effect on charcoal rot infection was inconsistent. The results of the field and greenhouse

studies were contradictory.

Recent developments of tissue culture techniques in soybeans made possible an attempt of in vitro selection of resistance to the toxin of M. phaseolina. In this study, we evaluated the variation of callus response to the toxin among selected cultivars. Bay showed more susceptibility than all other cultivars in terms of callus growth rate reduction and browning scores on medium containing culture filtrate of the fungus. Douglas seemed to be more susceptible than Sprite and Williams 82, which agreed with the results of field root colonization studies. Since the toxin may play a very important role in the diseasing process, in vitro selection may prove to be a feasible approach to utilize such an in vitro variation to reduce soybean charcoal rot damage.